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Fitness Costs of Fluoroquinolone Resistance in *Streptococcus pneumoniae*▽

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The fitness cost of the genes responsible for resistance to fluoroquinolones in clinical isolates of *Streptococcus pneumoniae* were estimated in vitro in a common genetic background. Naturally occurring *parC*, *parE*, and *gyrA* loci containing mutations in the quinolone-resistance-determining regions were introduced by transformation into *S. pneumoniae* strain R6 individually and in combinations. The fitness of these transformants was estimated by pairwise competition experiments with a common R6 strain. On average, single *par* and *gyr* mutants responsible for low-level MIC resistance (first-step resistance) impose a fitness burden of approximately 8%. Some of these mutants engender no measurable cost, while one, a *parE* mutant, reduces the fitness of these bacteria by more than 40%. Most interestingly, the addition of the second *par* or *gyr* mutations required for clinically significant, high-MIC fluoroquinolone resistance does not increase the fitness burden imposed by these single genes and can even reduce it. We discuss the implications of these results for the epidemiology of fluoroquinolone resistance and the evolution of acquired resistance in treated patients.

Streptococcus pneumoniae remains a leading cause of invasive bacterial diseases such as pneumonia, meningitis, and sepsis and is the most significant cause of acute otitis media in children, accounting for between 5 and 7 million otitis media cases annually in the United States alone (9, 48). While in the past, these infections were effectively treated with beta-lactam antibiotics including penicillin, in part due to the rising frequency of resistance to these compounds (32, 37, 45), other classes of antimicrobial agents are increasingly being employed to treat pneumococcal disease. Of these, the fluoroquinolones have been particularly successful for treating pneumococcal infections in adults (30).

Unfortunately, as observed for antimicrobial agents of other classes, *S. pneumoniae* strains with high-MIC resistance to fluoroquinolones (FQs) have emerged (1, 7, 31, 51) and lead to a number of reported instances of FQ treatment failure (11, 13, 41). Although the frequency of clinical isolates of *S. pneumoniae* with high-MIC resistance to FQ remains low and appears to have leveled off at ~3% for isolates within North America (28–30, 42), it is not clear if this frequency has genuinely plateaued or if it will continue to rise with increasing FQ use. This concern is particularly heightened by proposals to recommend FQs for a broader range of indications and for children, for which and for whom they are not currently employed (1).

In theory, whether or not the frequency of resistance to FQs or any other antimicrobial agent will increase, and the rate of that increase, will be directly proportional to the efficacy of the antimicrobial and the extent of its use and inversely proportional to the cost that resistance imposes on bacterial fitness (3, 25). In compartment models of antimicrobial treatment, the fitness of a bacterial strain is directly proportional to its rate of infectious transmission and its ability to compete with other strains within coinfected hosts and inversely proportional to the rate at which it is cleared from treated and untreated infected patients. If resistance imposes a fitness burden, these models predict that there are threshold levels of antimicrobial use below which the frequency of resistance will not increase, where the level of the threshold is directly proportional to the magnitude of the fitness cost (8, 24). If there is heterogeneity in the fitness of resistant strains, those with the highest fitness are anticipated to eventually prevail (8). Accordingly, to be able to predict whether antimicrobial resistance will increase at all and roughly at what rate, in addition to estimating the volume of drug use and drug efficacy, it is essential to determine both the average fitness costs of resistance and the variability in those costs. Ideally, with these models and realistic parameter estimates, it should be possible to develop antimicrobial use and restricted-use protocols to prevent or minimize the rate of ascent of resistance.

It is possible to obtain data on rates of antimicrobial use (6), but it is difficult or virtually impossible in humans and human populations to estimate the effects of resistance on the in vivo competitive performance of bacteria or the respective rates of clearance and infectious transmission in treated and untreated hosts of sensitive and resistant strains. A surrogate of these measures of the epidemiological fitness of antimicrobial-resistant strains is the relative fitness of susceptible and resistant bacteria in in vitro culture as measured by their competitive performance. Numerous studies have shown that by this criterion, resistance commonly engenders a fitness burden (2, 5, 17, 18, 34, 35). Less known from these studies is the range of variation in the fitness costs among different resistance-encoding genes. In a recent study, Gagneux and colleagues (15) used an in vitro pairwise competition protocol to estimate the magnitude of and variation in the fitness costs of rifampin resis-

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tance in *Mycobacterium tuberculosis* from a diverse set of clinically isolated and laboratory-generated mutant *rpoB* genes. Their results indicated that although some rifampin-resistance-determining *rpoB* genes from clinical strains of *M. tuberculosis* engendered substantial fitness costs (in excess of 40% reductions in fitness), some imposed little or no fitness burden (less than 5%).

In this study, we explore the in vitro fitness cost of mutations responsible for resistance to FQ in clinical isolates of *S. pneumoniae*. Unlike rifampin, for which high-MIC resistance can be achieved by point mutations in a single gene (44), clinically significant resistance to FQ in the pneumococcus requires mutations in at least two genes in the quinolone-resistance-determining regions (QRDR) (7, 20, 36, 42) of the topoisomerase IV genes (*parC* or *parE*) and in the DNA gyrase genes (*gyrA* or *gyrB*). By pairwise competition, we estimated the in vitro fitness effects of single *gyrA*, *parC*, and *parE* mutants, which in spite of causing only marginal increases in FQ MICs, are of clinical importance as the first step in the generation of high-MIC resistance (16, 19, 39). We then estimated the contribution to fitness of the second-step mutation at these loci. Our results show that while, on average, the single gene mutations responsible for low-MIC FQ resistance confer a significant fitness cost in *S. pneumoniae*, there is substantial heterogeneity in the magnitude of these costs. Included among these clinically isolated first-step FQ resistance mutations are some that engender no significant cost. Our results also suggest that the second mutations needed for high-MIC resistance to FQ contribute little additional fitness burden relative to that already attributable to the first-step mutations, and some appear to ameliorate this fitness burden. We discuss the implications of these results for the epidemiology of transmissible (primary) FQ resistance and the generation of de novo (acquired) resistance in patients infected with susceptible strains.

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MATERIALS AND METHODS

Strains for transformation. In order to generate a series of otherwise isogenic clones containing first-step and double QRDR mutations, we transformed *S. pneumoniae* strain R6, an unencapsulated derivative of serotype 2 strain D39 that is wild type in both *gyrA* and *parC* (methods are described below). Alleles of *parC*, *gyrA*, and *parE* containing single QRDR mutations were amplified from levofloxacin-resistant clinical isolates collected as part of the CDC Active Bacterial Core surveillance program and were characterized in detail previously (40) and are also listed in Table 1. As a common competitor for these transformants, we used a spontaneous streptomycin-resistant mutant of R6, called R6:Str^r.

MIC determination. MIC was determined by using either Etest or serial dilution. Etests were carried out, according to the manufacturer's instructions (AB-BIODISK, Solna, Sweden), on tryptic soy agar with 5% sheep blood agar plates incubated overnight at 35°C. Serial-dilution MICs were determined using Mueller-Hinton broth with 5% horse blood according to established Clinical and Laboratory Standards Institute (CLSI) protocols (34a). All assays were conducted twice. In order to test for increased efflux activity, assays for MICs of ciprofloxacin for transformants were estimated in the presence of reserpine using established protocols (4).

Transformation protocol. Strains containing *parC*, *parE*, and *gyrA* first-step and double mutants were constructed by transforming strain R6 with alleles from levofloxacin-resistant clinical isolates of *S. pneumoniae* containing single QRDR mutations (40). Primers and PCR cycling conditions specific to QRDR were used to amplify fragments from genomic DNA of these clinical isolates according to the protocol described previously by Pan et al. (38). Transformation was conducted according to established protocols (33). Putative first-step transformants were selected on Mueller-Hinton blood agar with either 1.5 μg/ml or 2 μg/ml ciprofloxacin or 0.75 μg/ml moxifloxacin. Single resistant colonies were randomly selected within 24 h, grown to an optical density at 630 nm (OD_{630}) of ~0.3 in Todd-Hewitt–yeast (THY) broth, and stored in 200-μl aliquots at 80°C. Double mutant clones containing two QRDR mutations were generated by transforming each first-step mutant with amplified PCR products from each other first-step clone. These high-MIC transformants were selected on agar containing either 8 μg/ml ciprofloxacin or 2 μg/ml moxifloxacin, concentrations that prevented the growth of first-step mutants. In order to confirm the identity of the introduced mutations in the transformants, QRDR regions of first-step and double mutants were amplified by PCR and sequenced.

TABLE 1. Strains used in this study or as a source of genomic DNA for transformation

Strain	Relevant genotypic feature(s)	Source or reference
R6	Unencapsulated derivative of D39 wild type at *parC*, *parE*, and *gyrA*	Laboratory isolate
R6:Str^r	Spontaneous streptomycin-resistant strain	This study
94	*parC*(S79F)	42
349	*parC*(S79Y)	42
352	*parC*(D83Y)	42
85	*parE*(D435N)	42
88	*gyrA*(S81Y)	42
97	*gyrA*(E85K)	42
107	*gyrA*(S81F)	42

Competition experiments and fitness estimation. Pairwise competition experiments were used to estimate the in vitro relative fitness of each FQ-resistant mutant using a modified protocol similar to that described previously (23). Transformants for each of the mutants and the otherwise isogenic R6:Str^r ancestor were taken from a −80°C freezer and grown separately in 2 ml Todd-Hewitt–yeast broth (containing [per liter] 30 g Todd-Hewitt broth and 5 g yeast extract) to an OD_{630} of ~0.3. Each culture was diluted 100-fold into 2 ml THY broth and again grown to an OD_{630} of ~0.3. These separate cultures were then mixed in a ratio of 1:1, diluted 1,000-fold into 2 ml of THY broth, and grown together for ~10 generations of competitive growth, corresponding to a ~1,000-fold increase. The initial and final densities of the competing strains were estimated from CFU data by diluting and plating population samples onto THY blood agar with and without 100 μg/ml streptomycin. From these densities, we calculated the net population growth of each competitor during the course of the competition assay, also known as its Malthusian parameter (23). To estimate the relative fitness of each transformant, we used the ratio of the Malthusian parameter of the transformant to that of R6:Str^r. Five replicate competition experiments were performed for each genotype.

By definition, a fitness of 1 indicates that the mutation has no fitness effect or is selectively neutral. A ratio greater than or less than 1 indicates increased or decreased fitness, respectively. The magnitude of the fitness difference (e.g., 10%) corresponds to how much more rapidly (or slowly) one strain grew relative to the other during the course of the competition assay. To control for the effect of the Str^r marker, we estimated the fitness of the R6:Str^r common competitor relative to the R6 strain that we used for the transformation. The estimated relative fitness of R6:Str^r in this experiment is 0.98803 ± 0.018 (mean ± standard error), which is not significantly different from 1.

RESULTS

MICs. The MICs of the R6 transformants for five FQs are shown in Table 2. Consistent with the results of previous studies, the MICs of the first-step mutants are only slightly greater than that of the susceptible R6 parent strain. The MICs of the transformants carrying mutations in both *gyrA* and *parC*/*E* are significantly higher than those of either R6 or the first-step mutants and are in a range that meets the criteria of clinical resistance (9). One of the double transformants, [*parC*(D83Y) *gyrA*(S81F)] also showed evidence of an increase in efflux activity. All reported results exclude this clone, although its inclusion does not affect our conclusions.

TABLE 2. Identity of mutations and MICs of R6 transformants[a]

Mutation in *parC*	Mutation(s) in R6 transformants		MIC					
	*par*E	*gyr*A	CIP	CIP + RES	LVX	MXF	GAT	GEM
wt	wt	wt	1	0.5	1	0.25	0.12	0.03
S79F			2	2	2	0.25	0.12	0.06
S79Y			2	2	2	0.25	0.12	0.06
D83Y			3	2	2	0.25	0.12	0.06
	D435N		2	2	2	0.5	0.12	0.06
		S81Y	2	2	3	>0.5	0.25	0.12
		E85K	1	0.5	1	0.25	0.25	0.06
		S81F	1	1	1	0.5	0.25	0.12
S79Y		S81F	64	32	>32	8	≥4	1
S79Y		E85K	64	32	>32	8	≥4	1
S79F		S81F	64	32	>32	8	≥4	0.5
S79F		S81Y	64	32	>32	4	≥4	0.5
S79F		E85K	32	32	>32	8	≥4	1
D83Y		S81F	32	8	>32	4	4	0.12
D83Y		S81Y	64	32	>32	4	≥4	0.25
D83Y		E85K	64	32	>32	4	2	0.5
	D435N	S81F	32	16	>32	4	2	0.25
	D435N	S81Y	64	32	>32	4	≥4	0.25
	D435N	E85K	<4	<4	2	0.5	0.25	<0.06

[a] Mutations refer to both the gene and the identity of specific mutations. wt, wild-type R6; CIP, ciprofloxacin; RES, reserpine; LVX, levofloxacin; MXF, moxifloxacin; GAT, gatifloxacin; GEM, gemifloxacin.

Fitness of first-step mutants. Figure 1 shows the mean and standard error of the estimated fitness of each first-step mutant. Because the strains are isogenic save for their QRDR loci, we can conclude that costs or benefits due to resistance are caused by the mutations at these loci.

Overall, these first-step FQ resistance mutations engender a significant burden ($P = 0.012$), with an average fitness cost of ~8%. Using analysis of variance, we found significant variations in fitness among the seven different QRDR mutations ($F_{6,29} = 16.038$; $P < 0.001$). In addition, the *parC*, *gyrA*, and *parE* first-step mutants examined varied in the magnitude of the cost that each gene imposes on these bacteria ($F_{2,29} = 34.214$; $P < 0.001$), although this heterogeneity in fitness cost is driven largely by the sole *parE* mutant, which had exceptionally low fitness. With the removal of this mutant, as classes, we found no significant fitness difference between the *parC* and *gyrA* first-step mutants, but there remained significant overall variance among the remaining six first-step mutants ($F_{5,25} = 3.695$; $P = 0.017$). Moreover, there was significant variation among the three *parC* mutants ($F_{2,11} = 7.508$; $P = 0.012$) but not among *gyrA* mutants ($F_{2,12} = 2.265$; $P = 0.154$).

Fitness of double mutants. We obtained transformants for 11 of the 12 possible pairwise combinations of the first-step mutations described above (Table 2). The relative fitnesses of these constructs are presented in Fig. 2. Across all double mutants, we found an overall cost of ~6% ($P < 0.001$). However, in contrast to first-step mutants, we failed to identify significant variations in fitness among these double, high-MIC FQ resistance mutants. Finally, we found no significant difference between the average fitness of the single (*par* or *gyr*) mutants and that of double (*par gyr*) mutants ($P = 0.441$), suggesting that the primary cost of FQ resistance is borne by the first-step mutation.

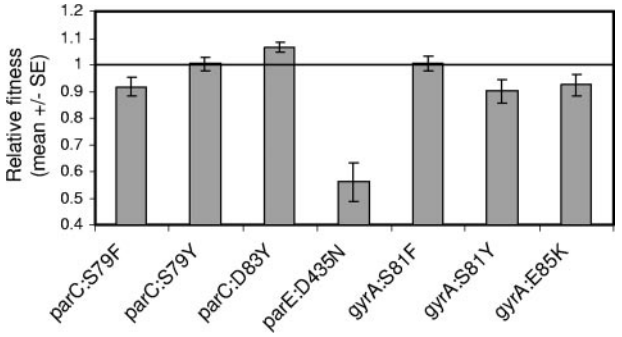

FIG. 1. Fitness of first-step mutants relative to R6:Str[r]. Mutations are indicated by gene and location of each mutation. Fitness values below 1 are costly, while those above 1 confer a fitness benefit.

DISCUSSION

FQs have proven to be effective for the treatment of invasive pneumococcal disease. However, as for other antimicrobial classes, mutations for resistance to FQ have increased in frequency and resulted in treatment failure (11, 13, 41). However, in contrast to other antimicrobial agents such as beta-lactams or macrolides, where the frequency of resistance has in some cases exceeded 50% of isolates (32), the frequency of FQ-resistant pneumococci remains low, on the order of 3% (28–30, 42) of isolates in the United States, and may well have stabilized at this frequency. Presumably, this can be attributed to the more restricted use of FQs versus alternative therapies for the treatment of invasive pneumococcus disease. However, if these drugs are approved for use in children, and for indications other than those for which they are currently employed, the rate of FQ use will increase, as will the selective pressure for resistance to this class of antimicrobial agents (1).

If resistant strains are substantially less fit than susceptible strains, in theory (3, 24, 25), with low and even modest rates of use of the selecting antimicrobial agent, resistance may not ascend, and if it does, the rate of increase will be low. Also, with some models of the epidemiology of antimicrobial treatment, the frequencies of resistance in communities could level off (25) and persist at low rates. From the perspective of the epidemiology of pneumococcal resistance to fluoroquinolones,

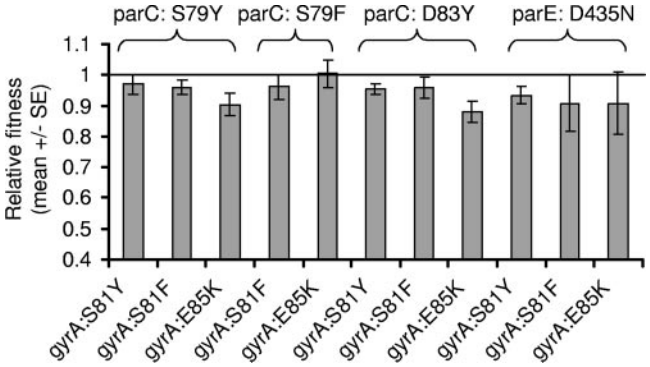

FIG. 2. Fitness of double mutants relative to R6:Str[r]. Mutations are presented in terms of the first step, shown above the bars. Second-step mutations are shown on the *x* axis. Fitness values below 1 are costly, while those above 1 confer a fitness benefit. SE, standard error.

we interpret the present results as being somewhat optimistic. As measured by their in vitro fitness in competition with otherwise isogenic strains, on average, the *gyr* and *par* mutations responsible for resistance in clinical isolates engender a significant, although small, disadvantage of approximately ~8% in first-step (low-MIC) mutants and ~6% for combination *gyr* and *par* mutants with resistance to high MICs.

Most interestingly, for the most costly single mutation studied here, *parE*, the addition of any of the three *gyrA* mutations studied restored the fitness of the bacteria to levels similar to those of the other high-MIC double mutants examined. Similar results have been observed for *parE* and *gyrA* double mutants in *Escherichia coli* (26). Presumably, the low fitness of the *parE* mutant, which may contribute to the rarity of this mutation among clinical samples of *S. pneumoniae* (43), can be attributed to a defect in the topoisomerase function coded for by this gene, perhaps affecting DNA segregation and potentially reducing the rate or fidelity of DNA synthesis (21, 49). A mechanistic explanation for how the altered gyrases coded for by these *gyrA* mutants interact with this defective topoisomerase to compensate for the pronounced fitness cost attributed to the *parE* mutation is an intriguing question that is beyond the scope of this report.

We qualify our optimism about the epidemiological implications of our experiments with the word "somewhat" because there are caveats to our interpretation. First, there is significant heterogeneity in the costs of first-step FQ resistance. Although four of the seven first-step mutants are costly, the other three, as well as pairs of *parC* and *gyrA* double mutants, exhibit no measurable fitness costs, at least as estimated in in vitro-paired competition assays. Should they emerge, the frequency of these no-cost mutants are anticipated to increase with little FQ use and eventually prevail (8). Second, for technical reasons (the need for otherwise isogenic backgrounds), our estimates of fitness were made on a genetic background different from that of the strains in which the mutations first arose. It is conceivable that as a consequence of epistatic effects with other genes in the genetic backgrounds of their origin, these *parC*, *parE*, and *gyrA* mutations engender an even lower (or greater) fitness cost than estimated here. Third, as noted in the introduction, competitive fitness as measured in vitro is only a surrogate of the epidemiological fitness of these bacteria. More specifically, it is not clear how this in vitro estimate of fitness costs translates into estimates of bacterial clearance in treated and untreated patients, in rates of infectious transmission relative to susceptible strains, or in their competitive performance in mixed infections in vivo. Finally, the epidemiological fitness of the pneumococcus is only somewhat affected by the specific loci considered here, those responsible for FQ resistance. While antimicrobial use is clearly affecting the fates of different lineages of pneumococci or other bacteria, other factors such as vaccine use (22, 46) can also play a role in the prevalence of drug-resistant clones. The impact of the second and third caveats can and will be addressed with subsequent work measuring the fitness costs associated with a broader range of FQ resistance genes and bacterial backgrounds and with in vivo competition within laboratory animals (19, 34, 47).

Implications for acquired resistance in treated patients. In terms of the generation of resistant strains in treated patients initially infected with fully susceptible bacteria, it could be argued that FQs have an advantage over antimicrobials for which clinical resistance can be achieved by single mutations. At least two separate genes (primarily *gyrA* and *parC* or *parE*) are required for clinically problematic, high-MIC resistance to FQ. Does a fitness cost in this first-step mutation substantially reduce the likelihood of acquired resistance to FQs?

The qualitative answer to this question is certainly affirmative. With respect to the issue of acquired resistance, we consider the results of this study to be even more optimistic than they are for the epidemiology and spread of primary resistance in the community. Fundamental to whether or not high-MIC resistance will be generated in a treated patient is the absolute number of first-step mutants at the time that treatment is initiated. If that number is high enough, double mutants for high-MIC resistance may be present at low densities or generated soon after the start of treatment, ascend to dominance during the course of treatment, and potentially lead to treatment failure (13, 14, 39, 41). If these first-step mutants engender a significant fitness cost, their numbers will remain low, too low for the generation of the second mutation needed for high-MIC resistance. And, as demonstrated previously (27) and as considered in more detail by Karl Drlica and colleagues (10, 12, 50) in their development of mutation prevention concentration strategies for antimicrobial treatment, if resistant mutants are not present at the start of treatment, with effective antimicrobial treatment, they are unlikely to be produced during the course of treatment. The costs carried by some of the mutations examined here should serve to further restrict the conditions for the emergence of resistant double mutants within hosts.

In spite of increasing FQ use, rates of resistance in pneumococci remain low globally and appear to have reached a plateau within North America (28–30, 42). The work presented here shows that fitness costs associated with the genes responsible for resistance to this class of antimicrobial agents can be sufficiently high to prevent the ascent of FQ-resistant pneumococci in areas where the use of these drugs is relatively low. However, included among these clinically isolated FQ resistance genes are those with low to negligible costs. If FQ-resistant strains with fitness costs of this low magnitude are prevalent, even modest use of FQs could be sufficient for them to not only increase in frequency but to continue to do so following FQ treatment. Surveillance is clearly needed to determine if the frequencies of resistant strains among clinical isolates correspond to the fitness costs of the mutations responsible for that resistance and to monitor the changes in the frequencies of FQ-resistant pneumococci carrying these mutations.

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